REVIEW

The Microbiome, Ocular Surface, and Corneal Disorders

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The ocular surface microbiome is an emerging field of study that seeks to understand how the community of microorganisms found on the ocular surface may help maintain homeostasis or can potentially lead to disease and dysbiosis. Initial questions include whether the organisms detected on the ocular surface inhabit that ecological niche, and, if so, whether there exists a core microbiome found in most or all healthy eyes. Many questions have emerged around whether novel organisms and/or a redistribution of organisms play a role in disease pathogenesis, response to therapies, or convalescence. Although there is much enthusiasm about this topic, the ocular surface microbiome is a new field with many technical challenges. These challenges are discussed in this review as well as a need for standardization to adequately compare studies and advance the field. In addition, this review summarizes the current research on the microbiome of various ocular surface diseases and how these findings may impact treatments and clinical decision-making. (Am J Pathol 2023, -:1e14; https://doi.org/10.1016/j.ajpath.2023.05.004)

Since the start of the human microbiome project in 2008, significant progress has been made to characterize the structure, function, and diversity of the healthy human microbiome.1 Although the ocular surface was left out of that landmark study, in part because of its low biomass environment, a growing body of evidence has accumulated, which delineates the structure and function of the ocular surface microbiome (OSM) in health and disease.2–18 The OSM refers to the community of microorganisms (primarily bacteria) that colonize the ocular surface. It is now well known that commensal microorganisms are required by humans for critical physiological roles, including regulation of immune tolerance, metabolism, and normal mucosal epithelial barrier function (Figure 1). Human microbial communities are also specific for each body site, and changes in the structure of these microbial communities may contribute to the pathogenesis of disease. A healthy microbiome is characterized by a diverse environment, whereas an abnormal microbiome (dysbiosis) may alter the homeostasis in favor of pathogenic bacterial invasion.3 The characteristics of the OSM and its relationship with ocular surface disease (OSD) is the focus of this review. Future investigations into the role of the microbiome in diseased states will continue to provide valuable insights into the pathophysiology of OSD and the potential for novel therapeutic targets.

The microbiome refers to a characteristic community of bacteria, fungi, and viruses that exist in a particular body niche. The microbiota describes the living organisms that make up the microbiome. These microbial communities are described and compared using measurements of diversity (different types of organisms), which can be qualitative or, more recently, quantitative. α-Diversity describes the diversity of organisms within a community, whereas β-diversity describes the difference in diversity between communities. In general, a healthy microbiome is characterized by a diverse environment.7,19,20


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The ocular surface includes the cornea, conjunctiva, eyelids, and the lacrimal and meibomian glands. The lacrimal functional unit is composed of the lacrimal gland (middle aqueous tear layer), the meibomian glands (outer lipid layer), the tear film, the conjunctiva with goblet cells (inner mucus layer), the cornea, and the neurointegration of these structures. \(^{21-23}\) (Figure 1). The lacrimal functional unit has evolved to maintain a clear cornea and defend the ocular surface from pathogens and toxic insults. There are three broad mechanisms that protect the ocular surface. These include mechanical mechanisms (blinking, mucins on the ocular surface, and intercellular tight junctions), chemical mechanisms (anti-inflammatory cytokines and antibacterial compounds (IgA and lysozyme)), and the immune response (antigen-presenting cells, B cells, and T cells) (Figure 1). A growing body of evidence suggests that the OSM should be added to the lacrimal functional unit.

The ocular surface is a low biomass niche, containing 0.06 bacteria per conjunctival cell versus the 10 bacteria per gut epithelial cell. \(^{19,21,24}\) Traditional cultures of the healthy ocular surface have reported 9% to 87% growth and demonstrate predominantly coagulase-negative *Staphylococcus* (CNS), *Cutibacterium* (previously *Propionibacterium*), and *Corynebacterium*. \(^{5,8,9,25-28}\) Next-generation sequencing (NGS) methods have increased the sensitivity, but also introduced many concerns about the accuracy and interpretation of the results. The organisms that make up the core microbiome (bacteria, fungi, and viruses) of the ocular surface play important roles in maintaining the normal homeostasis and determine the state of health or disease (Figure 1). There also exists immunoregulatory pathways between the gut microbiome and the eye. \(^{3,29}\) A better understanding of the composition and function of the OSM, as well as the role of the gut microbiome, will lead to a better understanding of the pathogenesis of OSDs. This review will address our current understanding of the OSM, the variability in sampling techniques, well-known concerns over NGS methods, and the potential role of the OSM in the pathogenesis of several ocular surface diseases.

### Measuring the Microbiome

Historically, bacteria were studied for their pathologic responses and for the development of therapeutic interventions. Currently, the focus is more on the qualities of the bacterial community (microbiome) and its role in local and systemic immune regulation and disease. This transition has occurred primarily because of low-cost high-throughput sequencing technology. \(^{9}\) Traditional culture methods involved swabbing the ocular surface and growing bacteria on agar plates. This conventional culture technique is restricted to fast-growing organisms that grow in the specific media and may limit organism identification. NGS methods, including 16S rRNA gene sequencing, dot hybridization assays, RNA sequencing, and shotgun metagenomics, in addition to traditional cultures, have provided additional data on the composition and proportions of the entire genome of the bacterial community. \(^{7,30-32}\) More importantly, these techniques do not distinguish between living organisms and nonvital genetic material. Because the ocular surface is an atypically low biomass environment, contamination is a major concern. \(^{9}\)

There are four major steps in microbiome investigations, each with its own set of concerns: i) sampling methods with potential contamination; ii) DNA isolation and potential reagent contamination; iii) library construction, which includes PCR amplification and potential sequencing errors; and iv) bioinformatic analysis and misattribution errors. To highlight the potential for contamination, >60 common contaminating taxa have been identified. \(^{20}\) There is also a recent trend to transition from reporting relative abundance to absolute abundance. \(^{33}\) Although the use of strict controls is imperative at each step to ensure the validity of results, Hornung et al. \(^{34}\) in a meta-analysis of NGS studies, found that only 30% of studies reported a negative control and 10% reported a positive control, which is similar to our survey of the articles we reviewed, where 47.5% did not report a negative control (Table 1).

### Sampling Methods

The optimal sampling method for ocular microbiome studies is currently not standardized and remains unclear. A variety of swab materials [nylon flocked, polyester (Dacron) wrapped, rayon wrapped, and foam tipped; Copan USA, Inc., Murrieta, CA), techniques (dry versus wet and firm versus gentle), and the presampling use of topical anesthesia, as well as other concurrent topical agents, have generated concern over the accuracy and interpretation of the results. Dong et al. \(^{6}\) reported different results using a dry cotton swab with firm pressure versus a moist cotton swab applied with minimal pressure. The former yielded higher abundance of Proteobacteria, whereas the latter yielded more *Staphylococcus* and *Corynebacterium* species. \(^{5}\) These results might be explained by a diluting effect of the moist swab or the minimal pressure technique detecting more transient organisms on the surface. Rinsing of the superficial organisms may also influence the results. \(^{2}\) Katzka et al. \(^{35}\) used 16S rRNA gene sequencing to compare the conjunctival microbiome results from three different swabs (calcium alginate, cotton-tipped applicator, and Weck-Cel cellulose sponge) just before an epithelial biopsy \((n = 48\) samples). They found that the calcium alginate swabs were the most representative of the microbiome determined from actual biopsy specimens. This disparity between swab versus biopsy results was also reported by Ozkan et al. \(^{11}\)

### Topical Anesthesia

Topical anesthesia, used to reduce sampling discomfort before swabbing, has introduced another important...
variable in interpreting results of both conventional and nontraditional techniques. Bactericidal effects of topical anesthesia can result from the disruption of the cell wall or cytoplasmic membrane, leading to cell lysis.\textsuperscript{22,36,37} Gram-positive organisms are more resistant to cell lysis because of the peptidoglycan in the cell wall.\textsuperscript{38} Shin et al\textsuperscript{37} reported in a laboratory setting a higher bacterial α-diversity of the conjunctiva in subjects without the use of topical anesthesia.

### Table 1 Summary of Sampling Techniques in 40 OSM Studies

<table>
<thead>
<tr>
<th>Laterality</th>
<th>Sampling tool</th>
<th>Contamination</th>
<th>Anesthetic</th>
<th>Region sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilateral sampling/unilateral</td>
<td>Cotton swab (40%)</td>
<td>Negative controls (37.5%)</td>
<td>Proparacaine (15%)</td>
<td>Conjunctival sac/inferior fornix (45%)</td>
</tr>
<tr>
<td>analysis (20%)</td>
<td>Isohelix swab (10%)</td>
<td>Negative controls and contaminant filtering (15%)</td>
<td>Benoxir (2.5%)</td>
<td></td>
</tr>
<tr>
<td>Unilateral sampling/unilateral</td>
<td>Dacron swab (5%)</td>
<td>Not specified (47.5%)</td>
<td>Repivacaine (7.5%)</td>
<td>Temporal canthus (5%)</td>
</tr>
<tr>
<td>analysis (37.5%)</td>
<td>FLOQ swabs (7.5%)</td>
<td></td>
<td>Tetracaine (2.5%)</td>
<td>Tarsal conjunctiva (5%)</td>
</tr>
<tr>
<td>Not specified (15%)</td>
<td>Multiple swabs (7.5%)</td>
<td></td>
<td>None (65%)</td>
<td>Bulbar conjunctiva (5%)</td>
</tr>
<tr>
<td></td>
<td>Other (12.5%)</td>
<td></td>
<td></td>
<td>Multiple areas (30%)</td>
</tr>
<tr>
<td></td>
<td>NA (17.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA, not available; OSM, ocular surface microbiome.
of a 50-μL drop of 0.5% proparacaine anesthetic versus those who received the topical anesthetic. Using proparacaine significantly altered both the microbial community composition as well as the structure (permutational analysis of variance \( P = 0.001 \)), as measured by unweighted/weighted UniFrac distances. Delbeke et al \(^{39} \) examined the role of anesthetic in a double-masked, prospective, randomized study of both eyes of 24 eligible volunteers undergoing general anesthesia. Preservative-free artificial tears were used in one eye, and preservative-free oxybuprocaine hydrochloride 0.4% was used in the other eye. A sterile nylon-flocked swab (FLOQSwabs; Copan, Brescia, Italy) was used to sample the inferior conjunctival fornix of each eye using a similar firm pressure. They found no significant difference in sequencing results between the two groups. In a subanalysis, they also found no difference between Gram-positive and Gram-negative organisms as well as between aerobic versus anaerobic organisms. Overall, roughly half of ocular microbiome studies report the use of topical anesthesia, \(^{7,9,35,40,41} \) whereas some do not (Table 1).\(^{16,19,30,31,42–45} \)

Concurrent Use of Other Topical (Glaucoma) Medications

Concurrent medications can also alter the microbiome results primarily through the toxic effects of preservatives. Chang et al \(^{46} \) examined 10 unilateral or asymmetric glaucoma patients and 7 controls with topical glaucoma medications placed in only one eye. They found greater \( \alpha \)-diversity in both eyes of patients with glaucoma receiving unilateral drops and greater relative abundance of Gram-negative versus Gram-positive organisms in controls. The authors speculated that the preservative benzalkonium chloride in the drops selectively inhibited growth of Gram-positive over Gram-negative bacteria.

CL Wear

Shin et al \(^{37} \) compared microbiome samples from contact lens (CL) wearers versus non-CL wearers. They obtained samples from the conjunctival fornix and the skin under the eye. They found that the microbial community of the conjunctiva and skin under the eye were more similar in CL wearers, presumably from repeated contamination on lens insertion. Compared with non-CL wearers, the conjunctiva of CL wearers also demonstrated a higher level of Pseudomonas, Acinetobacter, Methylobacterium, and Lactobacillus. Levels of Hemophilus, Streptococcus, Staphylococcus, and Corynebacterium on the conjunctiva were reduced in CL wearers compared with the non-CL wearers.

DNA Isolation Methods

Villette et al \(^{47} \) studied three DNA extraction protocols for low biomass samples, using serially diluted fecal samples, and found that silica columns gave better yield than bead adsorption or chemical precipitation. They noted that the starting material concentration is an important limiting factor. The methods worked best on samples with low to moderate amounts of contaminants. Prolonged mechanical lysing, silica membrane DNA isolation, and a seminested PCR improved the results for low biomass samples. The lower limit was found to be \( 10^6 \) bacteria/sample to give robust and reproducible results. They noted that whole shotgun sequencing requires \( 10^7 \) bacteria/sample and is therefore less suitable for low biomass samples. They also found that \( \alpha \)-diversity increased until \( 10^6 \) bacteria/sample and with mechanical lysing. Being consistent when taking samples is key to minimizing technical variation, they concluded.\(^{44} \)

Bioinformatics

Karstens et al \(^{48} \) compared four computational approaches to identify and remove contaminants, including filtering sequences present in the negative control, filtering sequences based on negative abundance, identifying sequences that have an inverse correlation with DNA concentration using Decontam, and predicting the sequence proportion in SourceTracker. They found that removing sequences found in the negative control erroneously removed >20% of the expected sequences. They concluded that all the methods, except the negative control filter, had accuracy >95%. However, our literature survey found 47.5% of OSM studies do not address contamination, representing another important variable to consider when interpreting microbiome results (Table 1).

Sample Laterality

Finally, laterality data are not routinely documented. In our own work, we found that approximately 50% of healthy controls (HCs) and patients have a similar microbiome in each eye, and 50% are dissimilar (as defined by a Bray-Curtis dissimilarity score >0.3).\(^{7} \) Cavouto et al \(^{49} \) also demonstrated similar \( \alpha \)-diversity between eyes. In our review of 40 publications, we found that 15% did not specify laterality, and 20% combined bilateral data, making interpretation difficult (Table 1). Only 27.5% of studies provided unilateral data from each eye, whereas the remaining studies reported unilateral data from one eye only. Given the low biomass environment, it is appealing to combine bilateral data to generate more positive findings. In our work, we added a whole genome amplification step to the 16S rRNA gene sequencing protocol to get unilateral resolution, but only 5% of reviewed studies included this step.
The Normal Core Commensal Microbiome of the Healthy Ocular Surface

Hamady and Knight\textsuperscript{50} proposed three different models for a core microbiome, including the following: i) substantial core, where most subjects share most of the taxa; ii) minimal core, where all the subjects share a few taxa; and iii) no core, where there are no taxa shared across subjects. The consistent presence of certain ocular surface taxa over time seems to support the possibility of an individual-specific or minimal core microbiome.\textsuperscript{12} Shade and Handelsman\textsuperscript{38} describe another model including a temporal aspect with five characteristic features of a core: i) membership: shared Operational Taxonomic Unit (OTU) occurrences across communities; ii) composition: similar OTU abundances across communities; iii) phylogeny: shared OTU lineage across communities; iv) persistence: OTUs shared over time within and across communities; and v) connectivity: OTUs covarying within a community and shared across communities. Does the normal healthy eye reflect the core microbiome? The organisms in the core are a subset of the organisms found in HC eyes, but so far studies do not agree on the existence of a core microbiome for the ocular surface (Table 2). Because many studies publish results from HC eyes, they do not necessarily reflect core microbiomes, which should be considered.

Traditional Culture-Only Studies

Willcox et al\textsuperscript{8} reviewed published studies of traditional cultures from the eyelid, conjunctiva, and tears. The number of conjunctival swabs that yielded no growth ranged from 9% to 87%, with the number of eyelid swabs yielding no growth between 0% and 48%. Variability resulted from differences in collection, transportation, and culture conditions. Overall, CNS, Cutibacterium, and Corynebacterium were the most common bacteria isolated from the conjunctiva, eyelids, and tears, with Cutibacterium and diphtheroid bacteria (mostly Corynebacteria) being relatively common.\textsuperscript{252} Suto et al\textsuperscript{57} cultured 579 patients before cataract surgery using traditional culture techniques with light swabbing of the inferior conjunctival fornix. Their positive growth rate was 39.2% with 191 (67% isolates) strains of Gram-positive cocci, 76 (26.7%) strains of Gram-positive bacilli, and 16 (5.9%) strains of Gram-negative bacilli. A total of 127 of the Gram-positive cocci (44.5% isolates) were methicillin-sensitive CNS, and 37 (12.7%) were methicillin-resistant CNS. All 76 (26.7%) Gram-positive bacilli were Corynebacterium.

Sequencing-Only Studies

Delbeke et al\textsuperscript{53} reanalyzed 359 publicly available sample sequences from HCs and found that Corynebacterium, Acinetobacter, Pseudomonas, Staphylococcus, Cutibacterium, and Streptococcus were most often identified. Corynebacterium was present in all publications (11/11) with a median of 10% (3% to 19%), followed by Acinetobacter (9/11; 6% ± 0.05), Pseudomonas (8/11; 19% ± 0.10), Staphylococcus (7/11; 6% ± 0.03), Cutibacterium (7%; 4% to 17%), and Streptococcus (3% ± 0.01) (both 5/11).\textsuperscript{53} Using 16S rRNA gene sequencing, Andersson et al\textsuperscript{16} also found Enhydrobacter and Brevibacterium, instead of Acinetobacter and Pseudomonas. They also demonstrated a core microbiome of Enhydrobacter, Brevibacterium, Staphylococcus, Streptococcus, and Cutibacterium (Table 2).\textsuperscript{16} At the genus level, the top 10 genera present were Pseudomonas, Enhydrobacter, Brevibacterium, Staphylococcus, Cutibacterium, Streptococcus, Acinetobacter, Corynebacterium, Chrysobacterium, and Micrococcus.\textsuperscript{16} Huang et al\textsuperscript{54} used Illumina (San Diego, CA) high-throughput sequencing technology to study conjunctival swabs of the upper and lower palpebral conjunctiva, caruncle, and fornix in 31 normal subjects. Most sequences contained 10 phyla with Proteobacteria (46.5%), Actinobacteria (33.89%), Firmicutes (15.5%), and Bacteroidetes (2.28%). At the genus level, they found Corynebacterium (28.22%), Pseudomonas (26.75%), Staphylococcus (5.28%), Acinetobacter (4.74%), and Streptococcus (2.85%). The relative abundances of prevalent genera varied significantly between individuals. Corynebacteria ranged from 0.32% to 79.28% between different subjects. Garza et al\textsuperscript{17} reported that the core conjunctival microbiota was composed of 12 genera: Pseudomonas, Cutibacterium, Bradyrhizobium, Corynebacterium, Acinetobacter, Brevundimonas, Staphylococci, Aquabacterium, Sphingomonas, Streptococcus, Streptomyces, and Methylobacterium. Ozkan and Willcox\textsuperscript{26} found Pseudomonas consistently present, and we did also, whereas Andersson et al\textsuperscript{16} found it higher in healthy eyes compared with patients with dry eye disease (DED).\textsuperscript{7}

Combined Traditional Culture and Sequencing Studies

In 2011, Dong et al\textsuperscript{6} studied the normal microbiome of four subjects using 16S rRNA gene sequencing and found the most common organisms to be Pseudomonas (20%), Cutibacterium (20), Bradyrhizobium (16%), Corynebacteria (15%), and Staphylococcus (4%). However, using traditional culture techniques, they found Staphylococcus, Cutibacterium, and Corynebacterium. Ozkan et al\textsuperscript{12} reported that traditional cultures resulted in Staphylococcus (46.5%), Cutibacterium (34.9%), Micrococcus (24.8%), and Corynebacterium (6.2%), whereas 16S rRNA gene sequencing resulted in the following phyla: Proteobacteria (74.4%), Actinobacteria (48%), Firmicutes (34.9%); and genera: Corynebacteria (39.5%), Sphingomonas (32.6%), Streptococcus (16.3%), and Actinobacteria and Anaerococcus (7%). Graham et al\textsuperscript{17} studied 91 normal eyes, and found 83% culture results of conjunctival swabs were positive, with CNS as the most common, with Staphylococcus.
**Table 2** Studies Looking for a Core Microbiome

<table>
<thead>
<tr>
<th>First author of study</th>
<th>Core microbiome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dong</td>
<td>No core exists at the species level</td>
</tr>
<tr>
<td>Ozkan</td>
<td>No core exists at the species level</td>
</tr>
<tr>
<td>Anderson</td>
<td>Core consists of <em>Enhydrobacter</em>, <em>Brevibacterium</em>, <em>Staphylococcus</em>, <em>Streptococcus</em>, and <em>Cutibacterium</em></td>
</tr>
<tr>
<td>Garza</td>
<td>Core consists of <em>Pseudomonas</em>, <em>Cutibacterium</em>, <em>Bradyrhizobium</em>, <em>Corynebacterium</em>, <em>Acinetobacter</em>, <em>Brevundimonas</em>, <em>Staphylococci</em>, <em>Aquaductbacterium</em>, <em>Sphingomonas</em>, <em>Streptococcus</em>, <em>Streptophyta</em>, and <em>Methylbacterium</em></td>
</tr>
<tr>
<td>Zhang</td>
<td>Core of DED and DED with DM group: unclassified <em>Clostridiales</em> and <em>Lactobacillus</em></td>
</tr>
</tbody>
</table>

DED, dry eye disease; DM, diabetes mellitus.

epidermidis in 100% samples. Molecular methods in 16 samples revealed *S. epidermidis*, *Rhodococcus*, *Corynebacterium*, *Cutibacterium*, as well as *Klebsiella*, *Bacillus*, and *Erwinia*.

Ocular Surface Biogeography

Ozkan et al.12 sampled four sites: i) skin, ii) eyelid margin, iii) fornix and limbal tissue from biopsy specimens during pterygium surgery, and iv) conjunctival surface. They found a significant difference in the bacterial community composition between the conjunctival surface and the fornix (*P* = 0.001) and limbal tissue (*P* = 0.001). However, they found no difference between the limbus and the fornix (*P* = 0.764) using fresh tissue specimens. Furthermore, the dominant genus was *Pseudomonas* (relative abundance of 79.9% in the fornix and limbal samples, whereas it was only 6.3% using the conjunctival surface technique).11 Cavuoto et al.10 studied subjects aged <18 years and found there were qualitative differences between three different sites. The Shannon Index was lowest in the conjunctiva compared with the eyelid margin and skin, suggesting mechanisms on the surface to control the richness and abundance of organisms.2,11 The variety of regions sampled also contributes to the heterogeneity of results (Table 1).

Age and Sex

The role of age and sex in the healthy OSM is currently unclear. Several studies found no significant role of sex on the richness or diversity of the healthy OSM, although Shin et al.37 did find differences in particular genera between the sexes in healthy eyes. On the contrary, age was found to have a strong effect on the microbial flora, with diversity increasing with age. Katzka et al.15 found that for corneal epithelial samples, β-diversity varied with age (>65 years), but α-diversity did not.

Geography and Diet

Deng et al.58 used 16S rRNA gene sequencing to study the microbiome in three different cities in China with different climates and diet. Beijing was in the north, where it is colder and dryer, with the population consuming more of a wheat diet. The coastal town of Wenzhou is more humid, and the people eat more fish. In the southern town of Guangzhou, where it is wet and warmer, people have a more rice-based diet. Their overall metagenomic results demonstrated 77% bacterial, 19.5% fungal, and 3% viral species. The most common bacterial organisms in their healthy control group were *Cutibacterium acnes* and *S. epidermidis*. Regionally, the most common organisms identified in subjects from Beijing were *Pseudomonas*, whereas in Guangzhou/Wenzhou, it was *C. acnes*. They also studied two groups in Guangzhou, where they identified a travel group who left the city for >15 days and a nontravel group who travels only to neighboring cities. They found that the travel group had a different composition than the nontravel group, suggesting that there might be environmental/dietary influences on the ocular microbiome. Altitude was also found to play a role on OSM composition.44

Fungal Elements

In addition to bacteria, NGS has expanded our understanding of fungal and viral elements found on the ocular surface, detecting organisms more frequently than traditional methods.59,60 In one study, conventional methods only detected *Aspergilli* in 12.5% of eyes, whereas NGS methods detected 65 genera in 73% of eyes. Another study found that five genera, *Malassezia* (74.65%), *Rhodotorula* (1.93%), *Davidiella* (1.89%), *Aspergillus* (1.25%), and *Alternaria* (0.61%), accounted for >80% of the fungal microbiome and was present in >80% of the individuals tested.60 They hypothesized that this might constitute a potential core fungal taxa on the normal ocular surface.

Temporal Stability

It is important to understand the temporal variability of the normal ocular surface microbiome to establish a baseline from which to determine the impact of disease or other environmental factors.12 Ozkan et al.12 studied 45 healthy subjects with ocular surface samples taken three times over a 3-month period without the use of topical anesthetic, to determine the temporal stability of the core microbiome using both culture and sequencing. The most detected taxon was *Corynebacterium* (11.1%), and no species was always
present in all subjects or in all subjects at any time. They concluded that although their results do not support a substantial core microbiome, as defined by Hamady and Knight, there might be an individual-specific core microbiome. Shin et al also determined that there were no differences in bacterial diversity between three different time points over 6 weeks in both CL wearers and non-CL wearers. Finally, Graham et al detected the same bacterial species over 3-month sampling on a small set of normal subjects (n = 4).

**Immune Regulation, Ocular Surface Dysbiosis, and Ocular Surface Diseases**

The role of the OSM in ocular surface disease pathophysiology and the association of dysbiosis with inflammation and infection have been the focus of much research (Figure 1). Generally, microbiome changes in disease seem to involve the following: i) a reduction in Gram-positive organisms; ii) a relative increase in Gram-negative organisms; and iii) a trend toward a decrease in α-diversity. de Paiva et al recently summarized our current understanding of the relationship between the ocular mucosal immune system and the surface microbiome. The ocular surface, unlike the inside of the eye, is not an immune-privileged site and requires specific mechanisms to induce immune tolerance. Follicular components of the conjunctiva function as immune surveillance structures with a variety of T cells (CD8⁺, innate lymphoid cells, natural killer cells, and γδ T cells), B cells, and dendritic antigen-presenting cells. Immune tolerance is mediated by activation of T-regulatory cells that down-regulate the immune response. The γδ T cells are also responsible for regulating host defense and inhibiting infectious keratitis by *Candida* and *Pseudomonas* through an IL-17 pathway (Figure 1). The antigen-presenting cells play important roles in ocular allergy, dry eye, and corneal transplant rejection reactions. Finally, there are innate lymphoid cells that secrete IL-13, an important cytokine for goblet cell activation and regulation (Figure 1). Goblet cell deficiency has been associated in animal models with dry eye. Any alterations in the multiple components above can lead to a disruption of the homeostasis of the ocular surface environment.

Ocular surface dysbiosis has been described in several systemic diseases with ocular surface manifestations [DED, diabetes, ocular graft-versus-host disease (GVHD), and Stevens-Johnson syndrome (SJS)] as well as several more local diseases [meibomian gland disease (MGD), allergic conjunctivitis, and bacterial keratitis].

**Dry Eye Disease**

Dry eye is “a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolality, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles.” Tear film instability, hyperosmolality, ocular surface damage, and ocular surface inflammation are key elements of the pathogenesis of DED (Figure 1). Liang et al hypothesis that conjunctival microbiota dysbiosis and a breakdown in the immune homeostasis on the ocular surface contribute to the development of DED. The loss of homeostasis on the ocular surface leads to a vicious cycle of hyperosmolality and tear film instability. There are three forms of dry eye: i) aqueous-deficient dry eye; ii) evaporative dry eye; and iii) mixed dry eye. The incidence of DED has been increasing every year, with a prevalence between 8.7% and 30.1%. Currently, there are three pillars of medical treatment: i) lubrication and epithelial protection, ii) management of inflammation, and iii) therapy for meibomian gland dysfunction.

Several studies have noted that bacterial culture positivity is higher in patients with DED, whereas one study found several species had an abnormally high abundance in DED. Hori et al looked at 67 women with DED and 56 controls. They found *C. acnes*, *CNS*, and *Staphylococcus aureus*, with 94% of samples being bacteria positive. Zhang et al found *S. epidermidis*, *Corynebacterium*, *Micrococcus*, and *Pseudomonas* in 78 patients with DED. *CNS, S. epidermidis*, *Corynebacterium*, and *Cutibacterium* have been identified as major genera/species in several studies. Lesser found genera include *Escherichia*, *Streptococcus, Bacillus, Rhodococcus*, and *Micrococcus*. In one study, α-diversity was significantly lower in DED compared with healthy eyes, as we have also shown.

Two studies also found that the α-diversity was higher for DED eyes than controls, although they had merged data from both eyes and used a unique rinsing method to sample the eyes, which may have led to differences.

**Dry Eye Disease versus Sjögren Disease**

There have been a multitude of human and animal studies investigating the role of both the ocular and gut microbiome on DED. Dry eye disease is the most frequent ocular manifestation of several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, and idiopathic Sjögren syndrome (SS). SS is a chronic autoimmune disease with dry mucosal surfaces and systemic muscular pain. Changes to the ocular environment could influence the bacterial composition. For example, patients with SS have low lactoferrin and lysozyme levels, two antimicrobial molecules that inhibit bacterial growth. In addition, the lacrimal gland becomes infiltrated with activated CD4⁺ T cells and B cells, and lymphocytic proliferation has been linked with Epstein-Barr virus infection, a common viral infection. These changes manifest as a decrease in the number of sterile cultures from SS eyes compared with healthy eyes, an
increase in *S. aureus*, and a decrease in diversity of SS eyes compared with healthy eyes.\textsuperscript{70,71,73} There is a discrepancy as to whether SS decreases the diversity compared with DED.\textsuperscript{18,73} The most abundant genera in SS eyes were *Actinobacter*, *Staphylococcus*, *Bacillus*, *Corynebacterium*, and *Clostridium sensus stricto 1*.\textsuperscript{70} 

**Dry Eye Disease and Diabetes Mellitus**

Zhang et al\textsuperscript{13} studied the OSM of patients with DED with and without diabetic mellitus compared with healthy controls. They found there were 10 genera that overlapped in the core microbiota group, whereas unclassified *Clostridiales* and *Lactobacillus* were the core microbiota of the DED and DED/diabetic mellitus groups, but not healthy controls.\textsuperscript{13} They found the DED with diabetic mellitus eyes had higher $\alpha$-diversity compared with healthy controls.\textsuperscript{13} Another study noted more CNS in diabetic patients and lower bacterial isolation in DED, although it increased in the group aged >60 years.\textsuperscript{27} A recent article by An and Zou\textsuperscript{74} proposed a mechanism for how an elevated ocular surface glucose environment may lead to hyperosmolarity and formation of advanced glycation end products and ocular surface dysbiosis, which result in ocular surface inflammation and disruption of the ocular surface homeostasis, leading to dry eye.

**Gut Dysbiosis and DED**

Gut dysbiosis has been reported in both human and animal models, with some variability between studies, including population/demographic factors, processing techniques, and other comorbid diseases. Increased abundance of *Blastocystis* and *Streptococcus* and reduced *Faecalibacterium* and *Prevotella* have been found in the patients with gut microbiome dry eye versus controls.\textsuperscript{75,76} de Paiva et al\textsuperscript{75} demonstrated in animal models that depletion of intestinal microbiome in antibiotic-treated mice significantly worsened the ocular surface response to desiccating stress environments. These mice also had a greater decrease in conjunctival goblet cells, more CD4\textsuperscript{+} infiltrating T cells in the conjunctival epithelium, and worsened corneal barrier function. Zaheer et al\textsuperscript{77} demonstrated that antibiotic-treated mice had increased lacrimal gland infiltration with lymphocytes and increased local inflammatory cytokines. Wang et al\textsuperscript{78} in a germ-free gut dysbiosis model, demonstrated increased inflammatory mediators on the ocular surface. Mendez et al\textsuperscript{79} evaluated the gut microbiome in 13 patients with Sjögren dry eye, 8 subjects with non-Sjögren dry eye, and 21 healthy controls. Gut microbiome 16S rRNA gene sequencing demonstrated Firmicutes as the dominant phylum (40% to 60%). Patients with dry eye had a relative depletion of Firmicutes and an increase in Proteobacteria.\textsuperscript{79} Payen et al\textsuperscript{80} studied gut microbiome signatures in patients following bone marrow transplant and compared those with acute onset GVHD versus those without and found a negative correlation between GVHD severity and anaerobic bacteria. Watane et al\textsuperscript{76} reviewed the role of gut microbiome and its associated compositional changes (dysbiosis) and immune-mediated dry eye, including primary Sjögren dry eye and secondary dry eye from ocular GVHD (oGVHD). In animal models, gut microbiome composition can modulate dry eye disease.

**Meibomian Gland Dysfunction**

MGD is an obstruction of the ducts that provide meibum to the ocular surface, leading to evaporative dry eye disease, blepharitis, and other conditions. The severity of MGD correlates with corneal fluorescein staining and tear film break-up time, which are parameters of ocular surface inflammation.\textsuperscript{81} Although one study found that the microbiome did not correlate with severity of MGD, others have found that as the severity increases, *S. epidermidis* decreased, *Corynebacterium* increased in moderate/severe MGD, and *Staphylococcus* increased in severe MGD.\textsuperscript{26,81,82} In addition, *Corynebacterium macginleyi* was only detected in MGD eyes, although only 26% of the MGD eyes were tested.\textsuperscript{26,81,82} The $\alpha$-diversity was similar between groups.\textsuperscript{81} Given these results, the authors believe evaluation of the bacterial severity on the ocular surface should play a role in treatment.

Dong et al\textsuperscript{81} studied 47 patients with varying degrees of severity of MGD and 42 healthy controls. The $\alpha$-diversity was similar between groups, whereas the $\beta$-diversity was similar within MGD severities, but higher than the HC group. There was a higher abundance of *Corynebacterium* in moderate/severe MGD and a higher abundance of *Staphylococcus* in severe MGD. The meibomian gland microbiome diversity decreases with age, which may explain the effect on homeostasis of the ocular surface.

**Ocular GVHD**

Shimizu et al\textsuperscript{13} used traditional culture techniques to study 32 eyes of 20 patients with oGVHD, 28 eyes of 20 patients after hematopoietic stem cell transplant without oGVHD, and 20 eyes from 11 healthy controls. They reported three species with traditional cultivation, and we identified all these taxa at their corresponding genus level in our study (*Staphylococcus*, *Cutibacterium*, and *Corynebacterium*).\textsuperscript{7,83} They found that the OSM is more diverse in patients with GVHD than patients without GVHD and HC, as we also found.\textsuperscript{7,83} They also found the oGVHD-severity score correlated with the number of species and the tear film break-up time. They concluded that the pathogenesis of GVHD is probably associated with changes in the OSM.

Andersson et al\textsuperscript{16} studied 39 patients with dry eye, with and without GVHD, and 28 healthy control subjects. They found that the $\alpha$-diversity decreased in dry eye and GVHD compared with healthy controls and that there was no difference between dry eye with and without oGVHD.\textsuperscript{16} They
also found no difference in sex or age. For the β-diversity, they found that dry eye and oGVHD were different from healthy controls and that the dry eye group did not differ from oGVHD. Again, there was no age or sex influence.

Stevens-Johnson Syndrome

Stevens-Johnson syndrome/toxic epidermal necrolysis are a spectrum of a rare, severe, adverse drug reaction that can result in significant ocular morbidity and systemic mortality. It can result in severe inflammation of the mucus membranes, including the ocular surface (cornea, conjunctiva, and eyelids). Patients who survive the acute disease often experience lifelong eye complications, including chronic ocular inflammation and severe dry eye, symblepharon, corneal scarring, ulceration, perforation, and blindness.84

Conventional culture techniques found that SJS eyes were positive more frequently than healthy eyes, which ranged from 58% to 95% for SJS eyes and 10% to 40% for healthy eyes.30,40,85,86 The most observed organisms were CNS, Corynebacterium, and Staphylococcus.80,85 More recently, Ueta et al45 found that SJS eyes clustered into four groups that are named by the predominant genera detected, including Corynebacterium 1, Neisseriaceae uncultured, Staphylocoecus, or a mix of Cutibacterium, Streptococcus, Fusobacterium, Lawsonella, and Serratia. They did find different species of Corynebacterium between healthy eyes and eyes of patients with SJS. The diversity of SJS microbiomes was reduced when compared with healthy eyes, as measured by the Shannon Index, which calculates diversity by including both the number of bacterial species detected and their abundance. Ueta et al45 found that the Shannon Index decreases from approximately 2.5 for healthy to approximately 1 for SJS, which is a similar finding to our study.7 In contrast, Kittipibul et al80 found an increase in the Shannon Index from 4 in healthy eyes to 6 in SJS eyes. They also observed an increase in the species observed in SJS eyes compared with healthy eyes, whereas Ueta et al15 and our study observed a decrease.7,40 The reasons for the discrepancies are unclear, although the studies did occur in different geographic regions and had technical differences.

Allergic Conjunctivitis, Atopic Keratoconjunctivitis, and Vernal Conjunctivitis

Several allergic reactions lead to chronic conjunctivitis, including allergic conjunctivitis (AC), allergic rhinoconjunctivitis, atopic keratoconjunctivitis, and vernal conjunctivitis (VKC), with the major mechanism of VKC and atopic keratoconjunctivitis being type IV hypersensitivity.74 AC is further broken down into seasonal AC and perennial AC. The top five genera in AC were found to be Bacillus, Staphylococcus, Corynebacterium, Acinetobacter, andRalstonia.87 A potentially distinguishing feature is the detection of Malassezia fungi on several diseased eyes.14,88 Brevibacterium aurantiacum and Staphylococcus sciuri were associated with seasonal AC/perennial AC. The change in diversity measurements is inconclusive.14,87–90

Inada et al89 studied 21 patients with VKC and atopic keratoconjunctivitis, who were treated with tacrolimus, and 6 healthy controls and divided the patients into mild and severe disease. There are three types of VKC: palpebral, limbal, and mixed. They found patients with allergic conjunctival disease to have significantly decreased α-diversity compared with healthy controls. The genera that were the most consistently different between healthy and allergic conjunctival disease eyes were Staphylococcus and Streptococcus, although Coprococcus, Ruminococcus, Veillonella, Delftia, Pseudomonas, and Moraxellaceae have also been found.14,91,92

The most abundant genus for patients with allergic rhinoconjunctivitis was Streptococcus, but like VKC, Moraxella, Corynebacterium, and Staphylococcus were also detected.90 Clinical scores in all subjects were negatively correlated with the Shannon Index, again suggesting that diversity increases, an eye becomes healthier.90

Bacterial Keratitis

Infectious keratitis describes the inflammation of the cornea due to microbes, such as bacteria and fungi. Contact lens wear is the leading risk factor.91 In a germ-free mice model, the presence of gut microbiota strengthened the ocular innate immune barrier to Pseudomonas by increasing the concentrations of immune effectors in the tear film, including secretory IgA and complement proteins.94 The protective immunity was found to be dependent on both eye and gut microbiota, with the eye microbiota having a moderate, but significant, impact on the resistance to infection.94 Genomic studies have noted an increase in pathogenic bacteria in patients with bacterial keratitis, most notably Pseudomonas, but also including Corynebacterium, Staphylococcus, Peptoniphilus, Sphingomonas, Paracoccus, Bosea, and Streptococcus.95–98 The conclusions on diversity are mixed, possibly due to differences in experimental design, such as the use of the V5 to V6 region or antibiotic use.95–98

Trachoma

Infection with Chlamydia trachomatis leads to trachoma, the leading infectious cause of vision loss worldwide.99 The disease leads to conjunctival scarring, trichiasis, corneal opacity, and blindness. Non-chlamydial bacterial species on the ocular surface are also significant factors in trachoma pathogenesis. Recent genomic studies have consistently found Cutibacterium, Cornebacterium, Staphylococcus, and Streptococcus, on the surface, with Pseudomonas, Acinetobacter, Micrococcus, Bacillus, Hemophilus, Neisseria, and Ralstonia found less frequently.42,43,55,100 Adults with scarring trachoma had reduced ocular bacterial diversity compared with healthy controls, with an increase in the relative abundance of
Corynebacterium, although another study found no difference in diversity.42,100

Treatments that Target the Microbiome

Characterization of the role of the OSM in OSD would also provide opportunities for targeted therapy. These might include antibiotics, diet, probiotics, prebiotics, topical microflora therapy, and fecal microbiota transplants.3,29,76,77,101,102 Many patients with OSDs have complex treatment regimens that complicate OSM analysis. The use of anesthetics, corticosteroids, antibiotics, and serum tears could all impact the bacterial community on the ocular surface. Thus far, one study did not observe an effect of artificial tears, whereas another saw no effect of topical anesthetics on the genera detected or α-diversity.4,39 They did suggest that Cutibacterium may be a discriminative biomarker for topical anesthetic use.59

Although prebiotics and probiotics have been studied, the results of larger meta-analyses have been inconclusive.3 In a mouse model, a mixture of Bifidobacteria, three Lactobacillus species, and Streptococcus partially improved experimental DED.85 Chisari et al103 conducted a clinical trial using oral Saccharomyces boulardii and Enterococcus faecium as probiotics for DED. Patients who received the probiotics reported a significant decrease in both frequency and severity of symptoms.103 Watane et al76 studied fecal microbiome transplants to treat DED symptoms in patients who meet the criteria for Sjögren disease. They found that effector and regulatory T-cell profiles were positively correlated with each other and symptom severity. They found no significant relationship between baseline gut microbiome and clinical metrics. The Dry Eye Questionnaire (DEQ5) and the Ocular Surface Disease Index (OSDI) surveys were negatively correlated with diversity indices, whereas five subjects reported improved symptoms. Finally, six of seven patients with vernal keratoconjunctivitis reported an improvement in symptoms of photophobia, itching, and tearing as well as sign of chemosis and injection following the use of topical Lactobacillus drops.101

Conclusions

Interpretation of the results of ocular surface microbiome studies can be challenging because of the many variables associated with characterizing this unusual paucibacterial environment. Table 1 outlines some of these variables, including the following: i) patient characteristics (age, sex, race, diet, environment/geography, comorbidities, and sample size); ii) sample size and study design; iii) culture techniques (traditional versus sequencing approaches); iv) concurrent topical medications (glaucoma, antibiotic, and corticosteroid) as well as anesthesia; v) sampling techniques [swab material, laterality, sampling pressure, and location (biogeography)]; and vi) processing protocols and analysis methods. As a result of this variability, there is still no consensus on what constitutes the core OSM and its stability over time, although consistent themes are emerging as specific genera are repeatedly observed, including Corynebacterium, Staphylococcus, Streptococcus, Pseudomonas, and Cutibacterium (Table 2). As methods continue to be optimized, and more studies are conducted using standardized protocols, we expect the results to provide more valuable data.

Characterizing the exact role of the microbiome in the normal homeostasis of the ocular surface and in the pathophysiology of OSD is the critical focus of most studies and the main thrust of this review. Figure 1 outlines the three major structural components of the ocular surface (OSM, the tear film, and the immune response), illustrates some of the known pathways for maintaining homeostasis, and highlights important aberrant pathways of dysbiosis and disease. In this review, we highlight the composition of the microbiome in various ocular surface diseases and discuss the interpretation of these results in the framework of the specific disease pathophysiology.

The OSM interacts directly or indirectly with all three structural components of the ocular surface, with commensal bacteria modulating the expression of inflammatory factors to maintain homeostasis. Corynebacteria inhibit overgrowth of pseudomonas through γδ T cells and an IL-17-mediated pathway.64 CNS can also inhibit pseudomonas proliferation through an IL-1β mechanism.61 Corynebacterium also stimulate mucin 1 (MUC1) and mucin 4 (MUC4) expression, which stabilizes the tear film. Immune tolerance to antigens occurs through the innate system through pattern recognition receptors and toll-like receptors. The adaptive response occurs through local B-cell, T-cell, and dendritic cell pathways. The physical barriers of tight junctions also maintain protection. The goblet cells secrete mucins as well as retinoic acid and transforming growth factor-β2 and IL-10, which activate immune tolerance.

A reduction in the α-diversity is commonly associated with OSD, including GVHD, DED, SJS, allergic conjunctivitis, and MGD. The α-diversity decreased in dry eye and GVHD compared with healthy controls, and there was no difference between dry eye with and without oGVHD. Higher α-diversity has been reported in some diseases, such as GVHD, potentially from immunosuppressive regimens that lead to increased pathogenic bacteria and stimulate the innate T-cell–mediated response.

The role of the gut microbiome on OSD also supports an interaction between the two. Following bone marrow transplant, there was a negative correlation between GVHD severity and gut anaerobic bacteria.60 In a germ-free mouse model, the presence of gut microbiota strengthened the ocular innate immune barrier to Pseudomonas by increasing the concentrations of immune effectors in the tear film, including secretory IgA and complement proteins.78,94
Depletion of intestinal microbiome in antibiotic-treated mice significantly worsened the ocular surface response to dry eye environment. Watane et al studied fecal microbiome transplants to treat DED symptoms in patients who meet the criteria for Sjögren disease. They found that effector and regulatory T-cell profiles were positively correlated with each other and with symptom severity.

In MGD, although some studies found that the microbiome did not correlate with severity of MGD, others have found that as the severity increases, α-diversity and S. epidermidis decreased, Corynebacterium increased in moderate/severe MGD, and Staphylococcus increased in severe MGD. The high level of Staphylococcus species can lead to tear film disruption through increased lipase activity and cytolysis from secreted α toxins (Figure 1). The bacterial community structures in MGD with DED may also be different than MGD only and controls.

Studies of OSM in DED support higher α-diversity due, in part, to damage to the ocular surface epithelial cells and reduction in mucins and antibacterial components in the tear film. Staphylococcus aureus releases α toxins, causing cytolysis and inflammation. Others found lower α-diversity, perhaps caused by sampling techniques. CNS, the most abundant commensal on the ocular surface, can up-regulate the expression of IL-1β, which can also induce dendritic cell maturation and immune activation and inflammation. The altered OSM in patients with DED and diabetes may result from high ocular surface glucose, hyperosmolarity, and high glycation end products, leading to immune activation and inflammation (Figure 1).

Finally, in patients with SJS, conventional culture techniques were positive more frequently than in healthy eyes. The most observed organisms were CNS, Corynebacterium, and Staphylococcus. Using modern sequencing techniques, the diversity of the SJS microbiome was also reduced when compared with healthy control eyes.

Looking Ahead

Many critical questions (and potential therapeutic targets) remain regarding the specific impact of the core microbiome not only on the regulation of the innate and adaptive immune responses, but also in the pathophysiology of ocular surface disease. How does the normal tear film interact with the microbiome to maintain immune tolerance? How does the loss of microbiome diversity affect the host response to infection? Figure 1 is an attempt to highlight and characterize our current understanding of these complex overlapping pathways that maintain the normal ocular surface homeostasis as well as those pathways that lead to inflammation, dysbiosis, and disease. For a more complete understanding of the exact mechanisms, future research will need to integrate a wide variety of variables, including the following: i) host factors (genetics, sex/age/race, ongoing treatments, and local and systemic antibiotics), ii) systemic immunity, iii) local immunity, and iv) the gut microbiota. Additional input data will require host immune profiling (genomics, transcriptomics, proteomics, and metabolomics) as well as microbiomics (16S rDNA/metagenomics, metatranscriptomics meta-proteomics, and meta-metabolomics). Our current review has hopefully illustrated the variability in obtaining accurate microbiome data and challenges of interpreting the results. It also highlighted the need for standardization throughout the process. We look forward to an integrative multi-omic biology and analytic approach to the pathophysiology, which may be necessary to fully characterize the role of the microbiome in ocular surface disease.

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